

Supplementary Methods

Sequence of oligonucleotides used for shRNA targeting EGFR

EGFR shRNA were obtained from the Harvard RNAi consortium. The following oligonucleotides (forward primer) were used to create the shRNA constructs (targeted sequence is underlined). The reverse primers were 5'-AATTCAAAA (sense)-CTCGAG-(antisense)-3'.

EGFR shRNA A:

CCGGCGCAAGTGTAAGAAGTGCGAACTCGAGTTCGCACTTCTTACACTTGCG
TTTTTG

EGFR shRNA B:

CCGGAGAATGTGGAATACCTAAGGCTCGAGCCTTAGGTATTCCACATTCTCTT
TTTG

EGFR shRNA C:

CCGGGCTGAGAATGTGGAATACCTACTCGAGTAGGTATTCCACATTCTCAGCT
TTTTTG

EGFR shRNA D:

CCGGGCTGGATGATAGACGCAGATACTCGAGTATCTGCGTCTATCATCCAGC
TTTTTG

EGFR shRNA E:

CCGGCCCGTCGCTATCAAGGAATTACTCGAGTAATTCCTTGATAGCGACGGG
TTTTTG

Supplemental Figure Legends

Supplemental Figure 1. Quantification of signaling proteins following gefitinib treatment.

Quantification of p-EGFR, p-ErbB-3 and p-Akt in H3255 and H3255 GR cell lines following gefitinib treatment was performed as described in Methods. The data was normalized to total EGFR, ErbB-3 and Akt expression, respectively and is represented as percent of untreated lanes. For example following 1 μ M gefitinib treatment, p-EGFR, p-ErbB-3 and p-Akt is maintained at ~10% of untreated cells in H3255 GR while it is decreased to 1% of control in H3255.

Supplemental Figure 2 A new genetic variant is detected in the H3255 GR cDNA.

A cDNA fragment encompassing exons 20 and 21 was amplified using RT-PCR from H3255, H1975 and H3255 GR, digested with Surveyor and analyzed on the WAVE HS system (Methods). Shown are the HPLC tracings and the numbers represent the size of the separated fragments. A new 70 bp fragment is detected only in H3255 GR in an analogous position to one in H1975 (asterix) indicating a DNA heteroduplex at position T790. The tracing from H3255 (dashed line) is shown in the background in the lower 2 panels.

Supplemental Figure 3. Sequence tracings of wild type and T790M containing cDNA clone from H3255 GR. Forward and reverse sequence tracings are shown for a wild type (top) and 1 of the 6 T790M containing cDNA clones (bottom) isolated from H3255 GR. The location of the point mutation (C to T) is highlighted by an asterix.

Supplemental Figure 4. EGFR knockdown with lentiviral EGFR shRNA. EGFR shRNA constructs were infected into A549 or H3255 cells (Methods). EGFR expression was assayed by Western blotting 7 days following infection.

Supplemental Figure 5. The expression of L858R/T790M or Del/T790M, but not GFP maintains activation of the ErbB-3/PI3K/Akt pathway in H3255 cells. H3255 cells expressing the indicated EGFR mutants or GFP control were exposed to increasing concentrations of gefitinib for 12 hours and lysed as described in Methods. The resulting extracts were probed with the indicated antibodies. The H3255 cells expressing EGFR (L858R/T790M) or EGFR (Del/T790M) maintain abundant levels of p-EGFR, p-ErbB-3, p-Akt, and p-Erk 1/2 in the presence of gefitinib. In contrast to HCC827 expressing WT/T790M, H3255 cells maintain moderate amounts of p-EGFR, p-ErbB-3 and p-Akt which renders these cells partially resistant to gefitinib (Figure 3B).

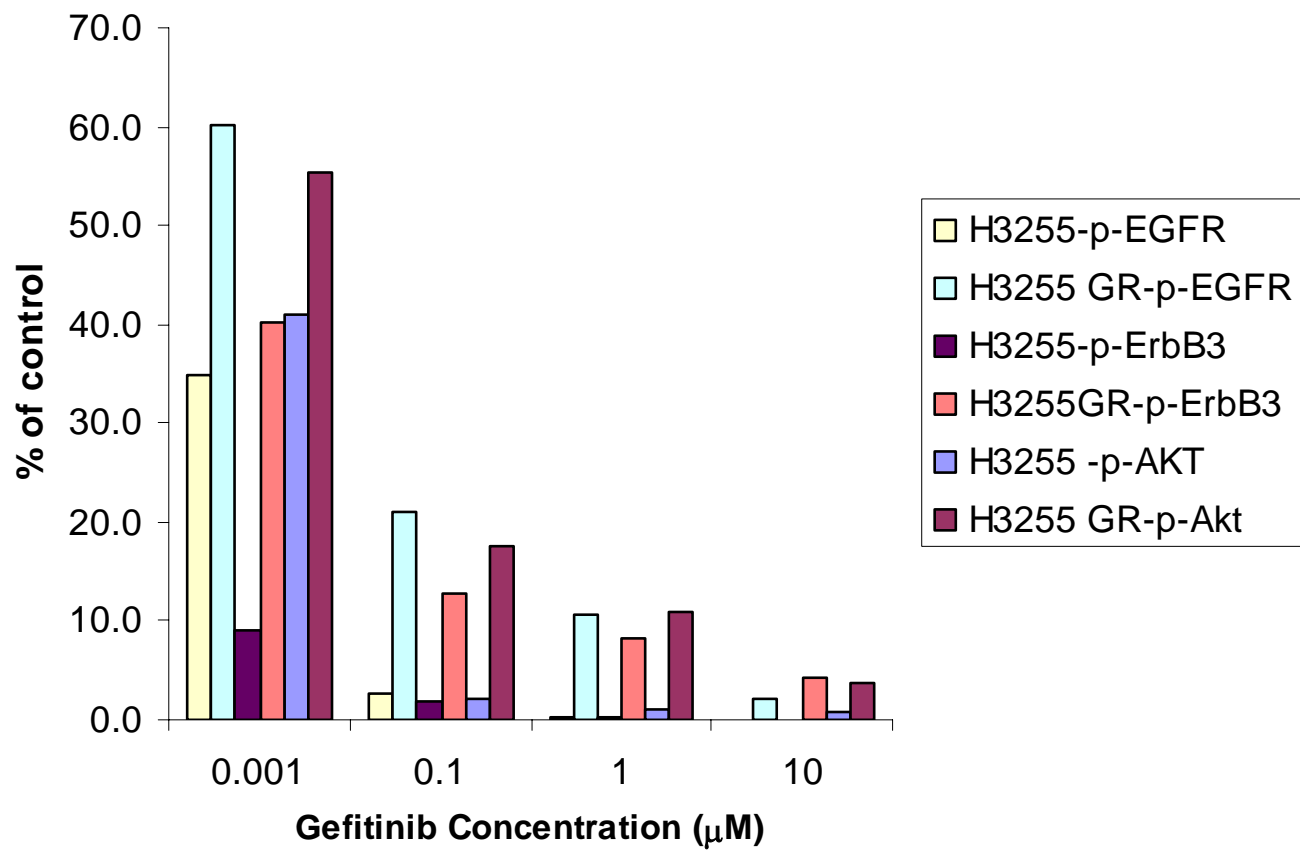
Supplemental Figure 6. Photographs of *nu/nu* HCC827 GFP and HCC827 Del/T790M xenografts. Xenografts from HCC827 GFP and HCC827 Del/T790M were generated as described in Materials and Methods. Control and gefitinib treated mice are photographed 30 days following initiation of treatment. The tumor injection sites are shown by the

hatched circles. There are no palpable tumors in the HCC 827 GFP mice. During the treatment 2 mice died in the HCC827 Del/T790M gefitinib treated group at days 11 and 18 of the study for unknown reasons. They showed no signs of weight loss or distress.

Supplemental Figure 7. HCC827 Del/T790M and L858R/T790M remain sensitive to the irreversible EGFR inhibitor, CL-387,785, and do not promote gefitinib resistance to surrounding cells via a paracrine effect.

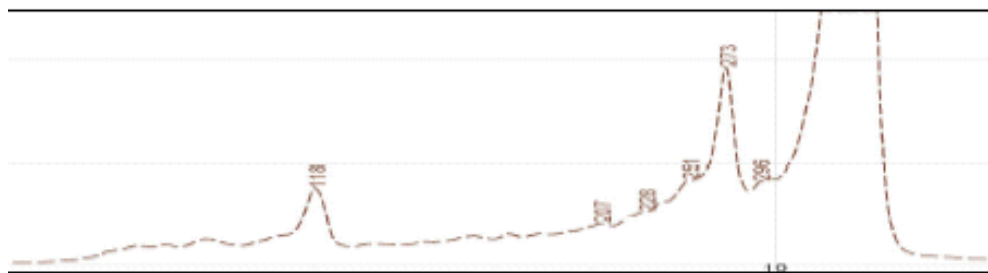
A. The HCC827 cells expressing EGFR (L858R/T790M) or (Del/T790M) remain sensitive to the irreversible EGFR inhibitor, CL-387,785. The HCC827 cell lines were subjected to an MTS survival assay in the presence of increasing concentrations of CL-387,785 (CL) and gefitinib (Gef). Cell lines expressing EGFR (L858R/T790M) or (Del/T790M) are significantly more sensitive to CL-387,785 as compared to gefitinib.

B. Co-culture of HCC827 (Del/T790M) with HCC827 (GFP) does not lead to resistance of HCC827 (GFP) cells. The HCC827 (Del/T790M) and HCC827 (GFP) cell lines were seeded together in different ratios to determine if a minor percent Del/T790M (D/T) cells could confer resistance to cells with no T790M mutation. Cells were seeded in the indicated ratios of HCC827 GFP cells and HCC827 Del/T790 (D/T) cells. Please note that 100% GFP cells remain sensitive, and 100% Del/T790M cells remain resistant. Co-cultures consisting of 50% (GFP:D/T (50:50)) of the Del/T790M cells develop intermediate resistance.

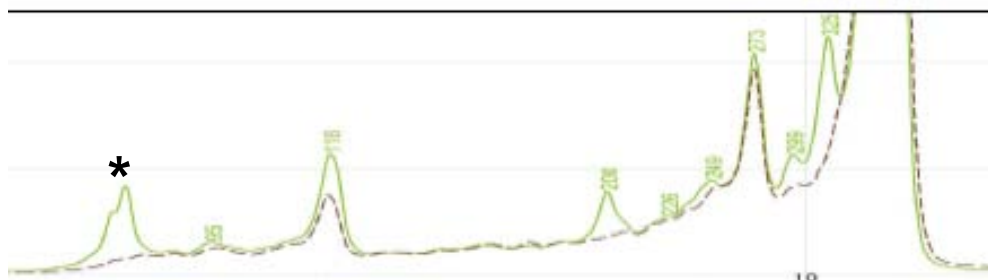


Supplemental Figure 1

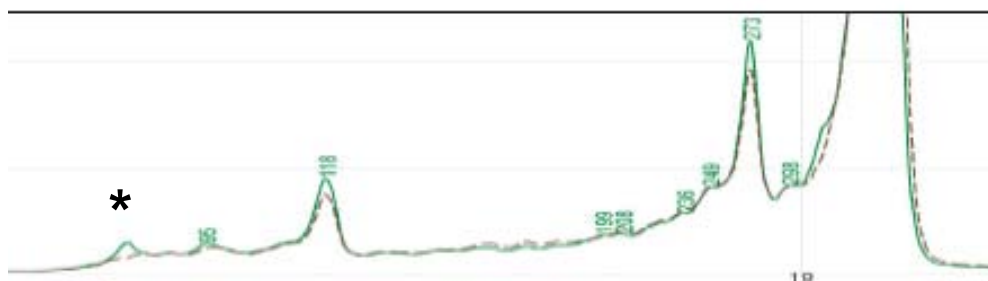
H3255



H1975

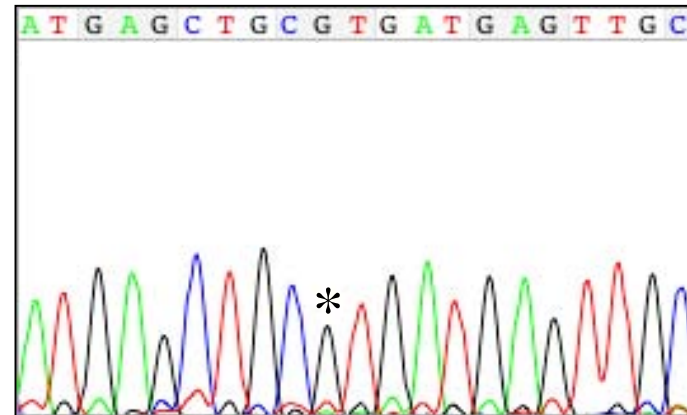
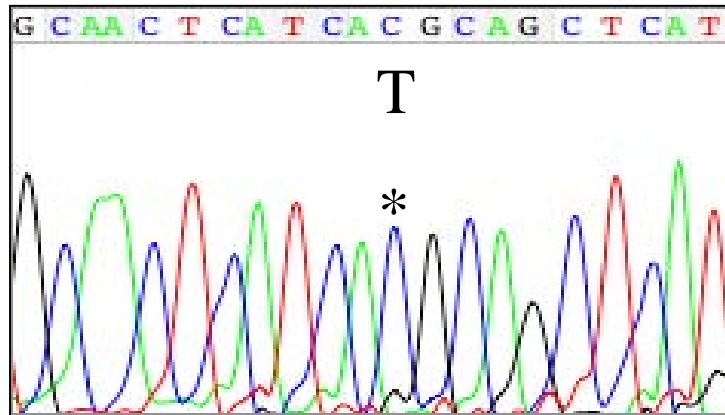


H3255 GR

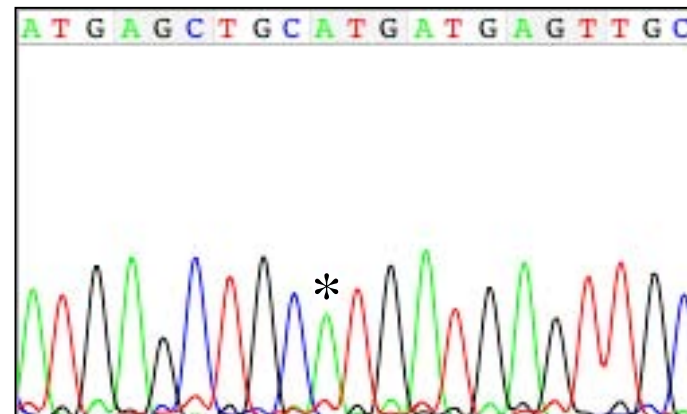
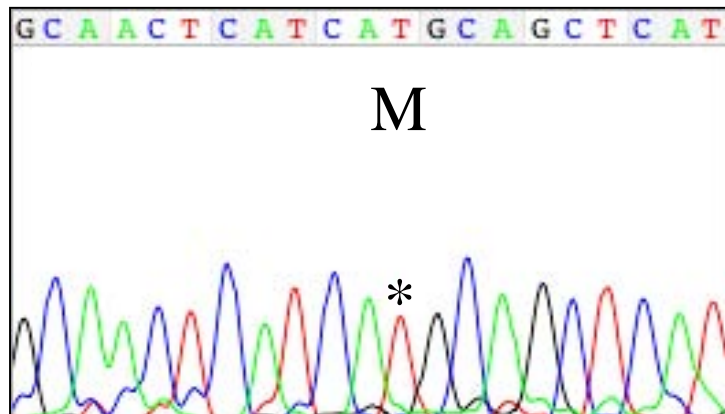


Forward

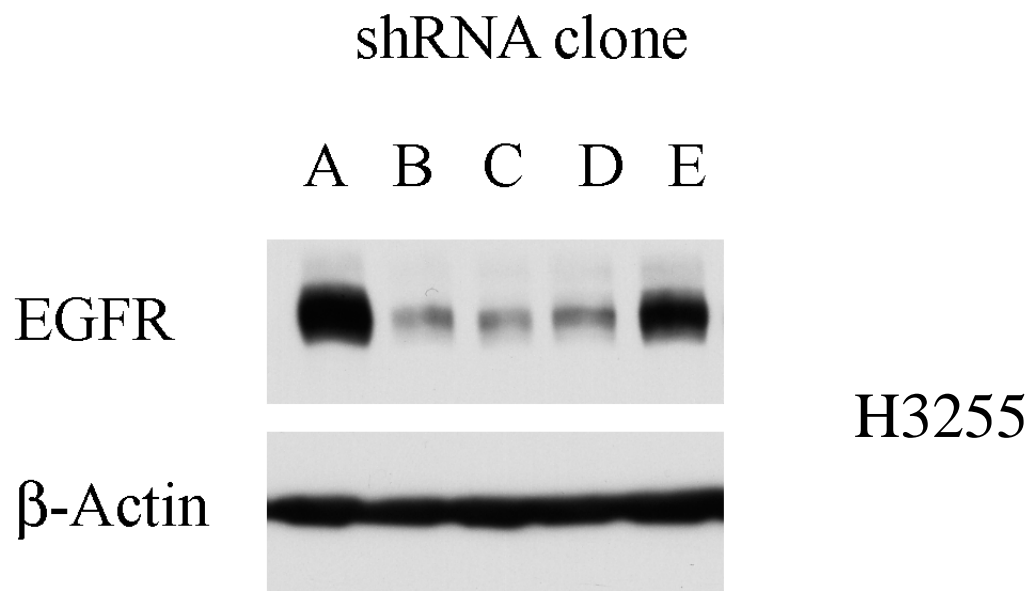
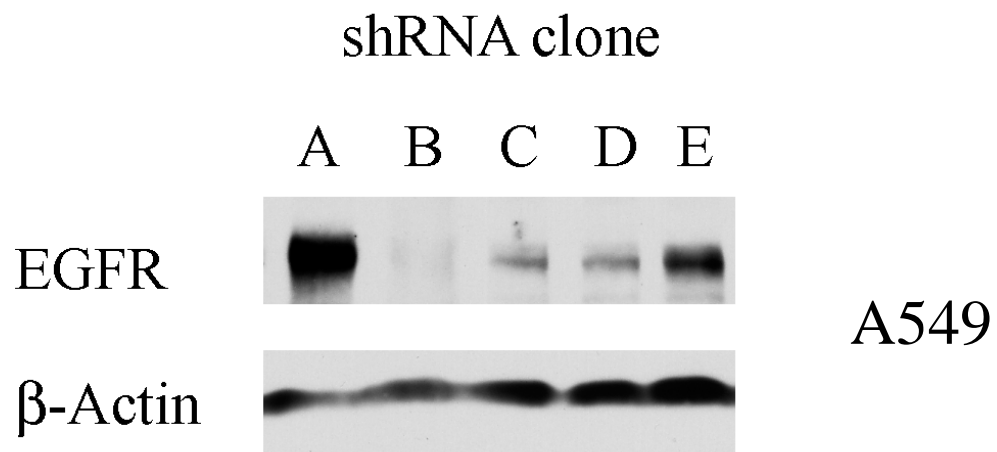
Reverse



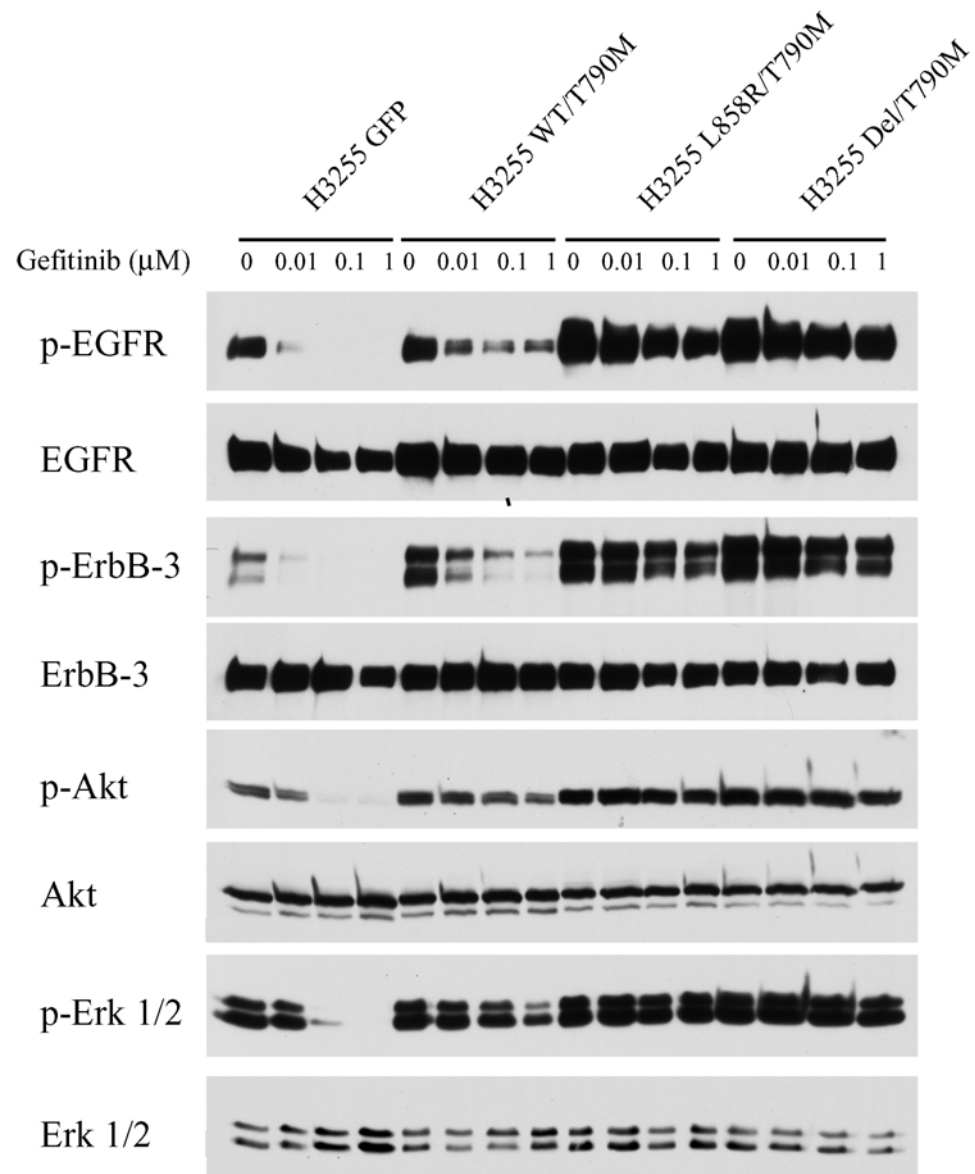
T790 (WT)



T790M



Supplemental Figure 4

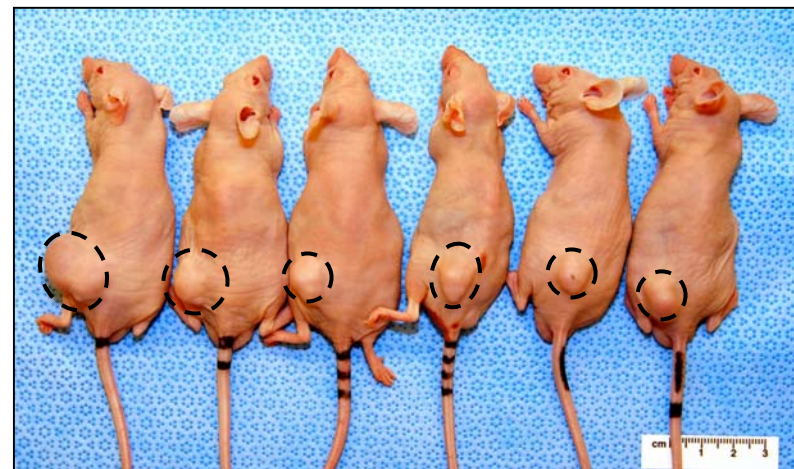
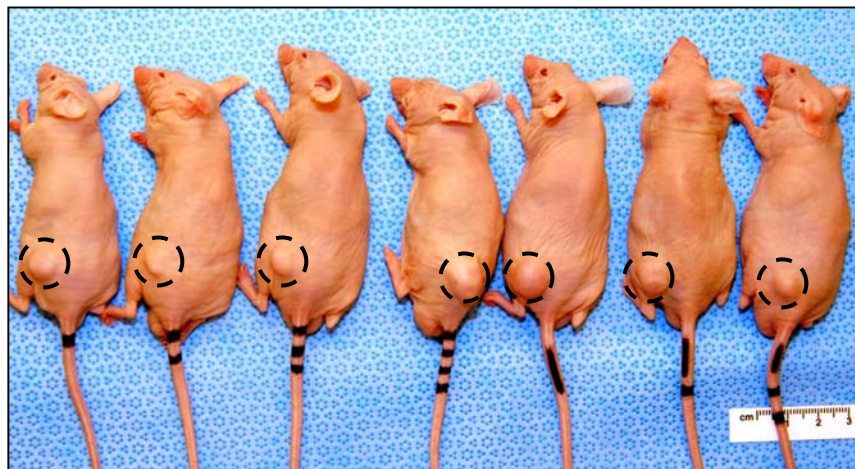


Supplemental Figure 5

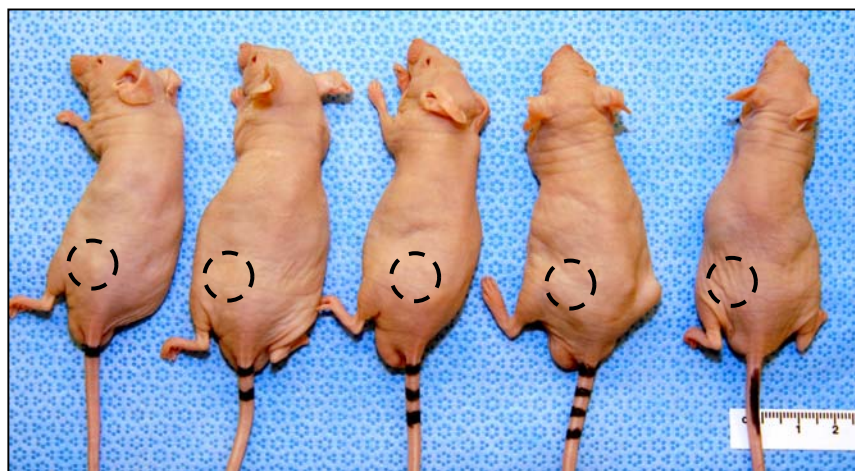
HCC827-GFP

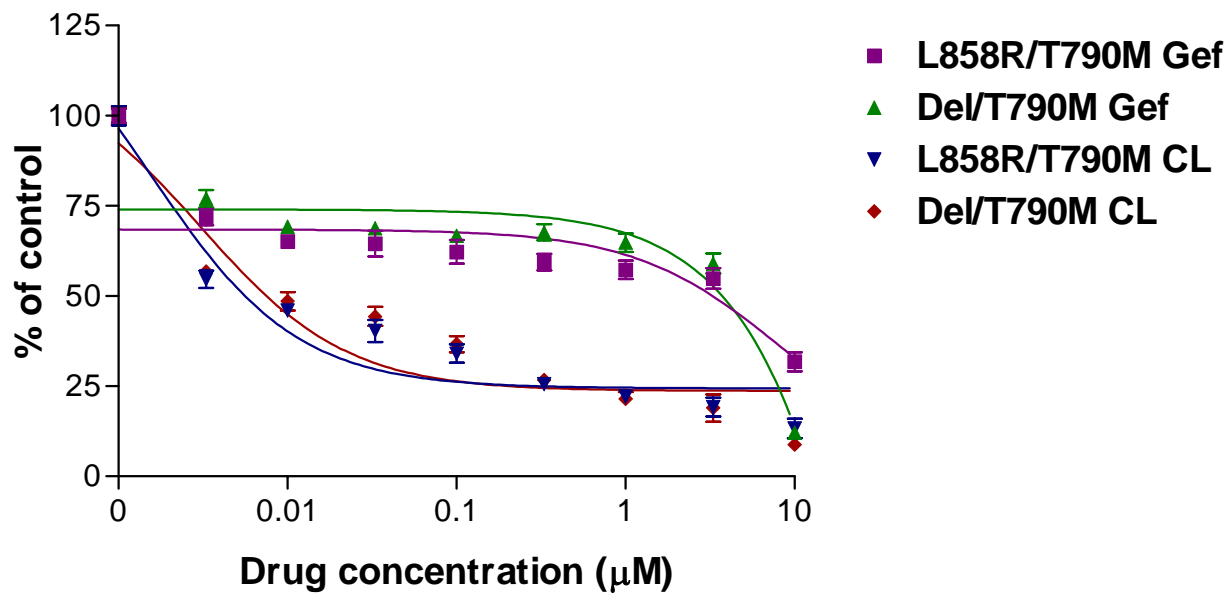
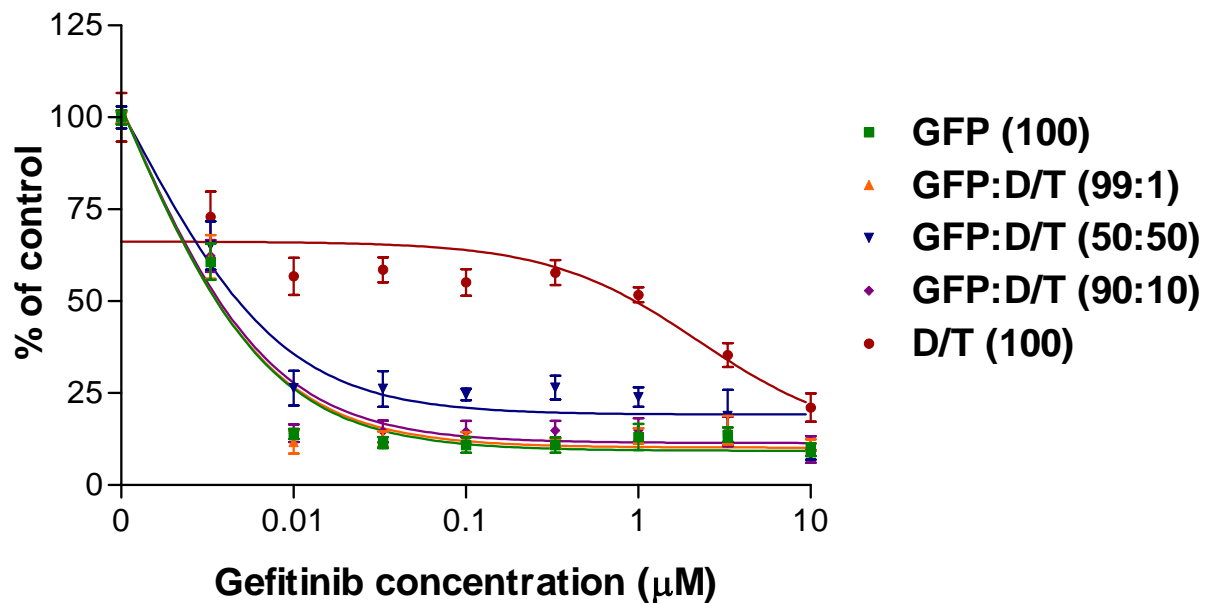
HCC827-DeI/T790M

Control



Gefitinib



A.**B.**

Cell line	Fold (+/-SD) increase in EGFR
H3255 GFP	N/A
H3255 WT/T790M	1.67 (1.52-1.85)
H3255 L858R/T790M	2.04 (1.61-2.59)
H3255 Del/T790M	1.56 (1.23-1.99)
HCC 827 GFP	N/A
HCC 827 WT/T790M	1.74 (1.24-2.43)
HCC 827 L858R/T790M	1.26 (1.15-1.39)
HCC 827 Del/T790M	1.36 (0.84-2.16)

Supplemental Table 1. Fold increase in total EGFR expression in transfected H3255 and HCC827 cell lines. EGFR expression was measured using real time PCR (Methods) and shown as fold increase compared to EGFR expression in the corresponding GFP transfected cell lines. SD; standard deviation, N/A; not applicable.